# Embryonic stem cell lines derived from human blastocysts

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**Abstract:**
*Human blastocyst-derived, pluripotent cell lines are described that have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages.*

**Full Text:**


**[Headnote]**
James A. Thomson,* Joseph Itskovitz-Eldor, Sander S. Shapiro, Michelle A. Waknitz, Jennifer J. Swiergiel, Vivienne S. Marshall, Jeffrey M. Jones

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Human blastocyst-derived, pluripotent cell lines are described that have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages. After undifferentiated proliferation in vitro for 4 to 5 months, these cells still maintained the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). These cell lines should be useful in human developmental biology, drug discovery, and transplantation medicine.

Embryonic stem (ES) cells are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation in vitro (1, 2). In chimeras with intact embryos, mouse ES cells contribute to a wide range of adult tissues, including germ cells, providing a powerful approach for introducing specific genetic changes into the mouse germ line (3). The term "ES cell" was introduced to distinguish these embryo-derived pluripotent cells from teratocarcinoma-derived pluripotent embryonal carcinoma (EC) cells (2). Given the historical introduction of the term "ES cell" and the properties of mouse ES cells, we proposed that the essential characteristics of primate ES cells should include (i) derivation from the preimplantation or permplantation embryo, (ii) prolonged undifferentiated proliferation, and (iii) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture (4). For ethical and practical reasons, in many primate species, including humans, the ability of ES cells to contribute to the germ line in chimeras is not a testable property. Nonhuman primate ES cell lines provide an accurate in vitro model for understanding the differentiation of human tissues (4, 5). We now describe human cell lines that fulfill our proposed criteria to define primate ES cells.

Fresh or frozen cleavage stage human embryos, produced by in vitro fertilization (IVF) for clinical purposes, were donated by individuals after informed consent and after institutional review board approval. Embryos were cultured to the blastocyst stage, 14 inner cell masses were isolated, and five ES cell lines originating from five separate embryos were derived, essentially as described for nonhuman primate ES cells (5, 6). The resulting cells had a high ratio of nucleus to cytoplasm, prominent nucleoli, and a colony morphology similar to that of rhesus monkey ES cells (Fig. 1). Three cell lines (HI, H13, and H14) had a normal XY karyotype, and two cell lines (H7 and H9) had a normal XX karyotype. Each of the cell lines was successfully cryopreserved and thawed. Four of the cell lines were cryopreserved

after 5 to 6 months of continuous undifferentiated proliferation. The other cell line, H9, retained a normal XX karyotype after 6 months of culture and has now been passaged continuously for more than 8 months (32 passages). A period of replicative crisis was not observed for any of the cell lines.

The human ES cell lines expressed high levels of telomerase activity (Fig. 2). Telomerase is a ribonucleoprotein that adds telomere repeats to chromosome ends and is involved in maintaining telomere length, which plays an important role in replicative life-span (7, 8). Telomerase expression is highly correlated with immortality in human cell lines, and reintroduction of telomerase activity into some diploid human somatic cell lines extends replicative life-span (9). Diploid human somatic cells do not express telomerase, have shortened telomeres with age, and enter replicative senescence after a finite proliferative life-span in tissue culture (1013). In contrast, telomerase is present at high levels in germ line and embryonic tissues (14). The high level of telomerase activity expressed by the human ES cell lines therefore suggests that their replicative life-span will exceed that of somatic cells.

The human ES cell lines expressed cell surface markers that characterize undifferentiated nonhuman primate ES and human EC cells, including stage-specific embryonic antigen (SSEA3, SSEA-4, TRA-1-60, TRA-181, and alkaline phosphatase (Fig. 3) (4, 5, 15, 16). The globo-series glycolipid GL7, which carries the SSEA-4 epitope, is formed by the addition of sialic acid to the globoseries glycolipid GbS, which carries the SSEA-3 epitope (17, 18). Thus, GL7 reacts with antibodies to both SSEA-3 and SSEA-4 (17, 18). Staining intensity for SSEA-4 on the human ES cell lines was consistently strong, but staining intensity for SSEA-3 was weak and varied both within and among colonies (Fig. 3, D and C). Because GL7 carries both the SSEA-4 and SSEA-3 epitopes and because staining for SSEA-4 was consistently strong, the relatively weak staining for SSEA-3 suggests a restricted access of the antibody to the SSEA-3 epitope. In common with human EC cells, the undifferentiated human ES cell lines did not stain for SSEA-1, but differentiated cells stained strongly for SSEA-1 (IS) (Fig. 3). Mouse inner cell mass cells, ES cells, and EC cells express SSEA-1 but do not express SSEA-3 or SSEA-4 (17, 19), suggesting basic species differences between early mouse and human development.

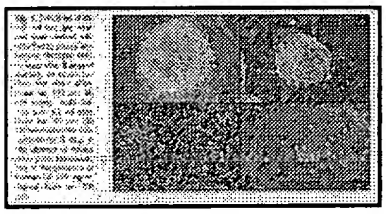

Fig 1. Derivation of the H9 cell line. (A) Inner cell mass-derived cells attached to mouse embryonic fibroblast feeder layer after 8 days of culture, 24 hours before first dissociation. Scale bar, 100 (mu)m. (B) H9 colony. Scale bar, 100 (mu)m. (C) H9 cells. Scale bar, 50 jim. (D) Differentiated H9 cells, cultured for 5 days in the absence of mouse embryonic fibroblasts, but in the presence of human LIF (20 ng/ml; Sigma). Scale bar, 100 (mu)m.

The human ES cell lines were derived by the selection and expansion of individual colonies of a uniform, undifferentiated morphology, but none of the ES cell lines was derived by the clonal expansion of a single cell. The uniform undifferentiated morphology that is shared by human ES and nonhuman primate ES cells and the consistent expression by the human ES cell lines of cell surface markers that uniquely characterize primate ES and human EC cells make it extremely unlikely that a mixed population of

precursor cells was expanded. However, because the cell lines were not cloned from a single cell, we cannot rule out the possibility that there is some variation in developmental potential among the undifferentiated cells, in spite of their homogeneous appearance.

The human ES cell lines maintained the potential to form derivatives of all three embryonic germ layers. All five cell lines produced teratomas after injection into severe combined immunodeficient (SCID)beige mice. Each injected mouse formed a teratoma, and all teratomas included gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm) (Fig. 4). In vitro, the ES cells differentiated when cultured in the absence of mouse embryonic fibroblast feeder layers, both in the presence and absence of human leukemia inhibitory factor (LIF) (Fig. 1). When grown to confluence and allowed to pile up in the culture dish, the ES cell lines differentiated spontaneously even in the presence of fibroblasts. After H9 cells were allowed to differentiate for 2 weeks, both (alpha)-fetoprotein (350.9 +/-14.2 IU/ml) and human chorionic gonadotropin (hCG, 46.7 +/-5.6 mIU/ml) were detected in conditioned culture medium, indicating endoderm and trophoblast differentiation (20).

Human ES cells should offer insights into developmental events that cannot be studied directly in the intact human embryo but that have important consequences in clinical areas, including birth defects, infertility, and pregnancy loss. Particularly in the early postimplantation period, knowledge of normal human development is largely restricted to the description of a limited number of sectioned embryos and to analogies drawn from the experimental embryology of other species (21). Although the mouse is the mainstay of experimental mammalian embryology, early structures including the placenta, extraembryonic membranes, and the egg cylinder all differ substantially from the corresponding structure of the human embryo. Human ES cells will be particularly valuable for the study of the development and function of tissues that differ between mice and humans. Screens based on the in vitro differentiation of human ES cells to specific lineages could identify gene targets for new drugs, genes that could be used for tissue regeneration therapies, and teratogenic or toxic compounds.

Elucidating the mechanisms that control differentiation will facilitate the efficient, directed differentiation of ES cells to specific cell types. The standardized production of large, purified populations of euploid human cells such as cardiomyocytes and neurons will provide a potentially limitless source of cells for drug discovery and transplantation therapies. Many diseases, such as Parkinson's disease and juvenile-onset diabetes mellitus, result from the death or dysfunction of just one or a few cell types. The replacement of those cells could offer lifelong treatment. Strategies to prevent immune rejection of the transplanted cells need to be developed but could include banking ES cells with defined major histocompatibility complex backgrounds or genetically manipulating ES cells to reduce or actively combat immune rejection. Because of the similarities to humans and human ES cells, rhesus monkeys and rhesus ES cells provide an accurate model for developing strategies to prevent immune rejection of transplanted cells and for demonstrating the safety and efficacy of ES cell-based therapies. Substantial advances in basic developmental biology are required to direct ES cells efficiently to lineages of human clinical importance. However, progress has already been made in the in vitro differentiation of mouse ES cells to neurons, hematopoietic cells, and cardiac muscle (22-24). Progress in basic developmental biology is now extremely rapid; human ES cells will link this progress even more closely to the prevention and treatment of human disease.

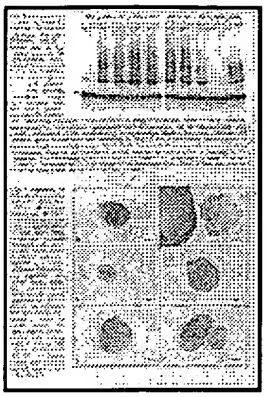

Fig. 2. Fig. 3.

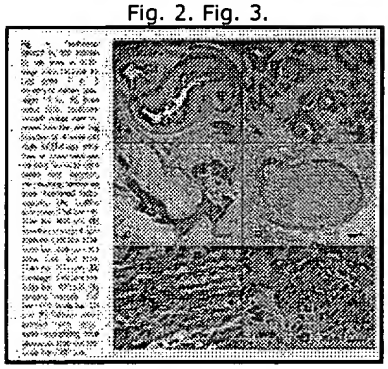

Fig. 4

## [Sidebar]

5 August 1998; accepted 7 October 1998

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Thirty-six fresh or frozen-thawed donated human embryos produced by IVF were cultured to the blastocyst stage in G1.2 and G2.2 medium (25). Fourteen of the 20 blastocysts that developed were selected for ES cell isolation, as described for rhesus monkey ES cells (5). The inner cell masses were isolated by immunosurgery (26), with a rabbit antiserum to BeWO cells, and plated on irradiated (35 grays gamma irradiation) mouse embryonic fibroblasts. Culture medium consisted of 80% Dulbecco's modified Eagle's medium (no pyruvate, high glucose formulation; Gibco-BRL) supplemented with 20% fetal bovine serum (Hyclone), 1 mM glutamine, 0.1 mM (beta)-mercaptoethanol (Sigma), and 1% nonessential amino acid stock (Gibco-BRL). After 9 to 15 days, inner cell mass-derived outgrowths were dissociated into clumps either by exposure to Ca^sup @^+/Mg^sup 2^+-free phosphate-buffered saline with 1 mM EDTA (cell line H1), by exposure to dispase (10 mg/ml; Sigma; cell line H7), or by mechanical dissociation with a micropipette (cell lines H9, H13, and H14) and replated on irradiated mouse embryonic fibroblasts in fresh medium. Individual colonies with a uniform undifferentiated morphology were individually selected by micropipette, mechanically dissociated into clumps, and replated. Once established and expanded, cultures

## [Reference]

were passaged by exposure to type IV collagenase (1 mg/ml; Gibco-BRL) or by selection of individual colonies by micropipette. Clump sizes of about SO to 100 cells were optimal. Cell lines were initially karyotyped at passages 2 to 7. 7. C. B. Harley, Mutat. Res. 256, 271 (1991). 8. H. Vaziri, C. M. Counter, R. C. Allsopp, Exp. Gerontol. 27, 375 (1992). 9. A. G. Bodnar et al., Science 279, 349 (1998). 10. L. Hayflick and P. S. Moorhead, Exp. Cell Res. 25, 581 (1961).

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16. Alkaline phosphatase was detected with Vector Blue substrate (Vector Labs). SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 were detected by immunocytochemistry with specific primary monoclonal antibodies and localized with a biotinylated secondary antibody and then an avidin or biotinylated horseradish peroxidase complex (Vectastain ABC system;

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20. hCG and a-fetoprotein were measured by specific radioimmunoassay (double AB hCG and AFP-TC kits; Diagnostic Products, Los Angeles, CA). hCG assays used the World Health Organization Third International Standard 75/537. H9 cells were allowed to grow to confluence (day 0) on plates of irradiated mouse embryonic fibroblasts. Medium was replaced daily. After 2 weeks of differentiation, medium in triplicate wells conditioned for 24 hours was assayed for hCG and a-fetoprotein. No hCG or a-fetoprotein was detected in unconditioned medium. 21. R. O'Rahilly and F. Muller, Developmental Stages in Human Embryos (Carnegie Institution of Washington, Washington, DC, 1987). 22. G. Bain, D. Kitchens, M. Yao, J. E. Huettner, D. I. Gottlieb, Dev. Biol. 168, 342 (1995). 23. M. V. Wiles and G. Keller, Development 111, 259 (1991).

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## [Author note]

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**Health and Medicine in the News**

**Headline:**

Cell-regrowth breakthrough could lead to new therapies.

**Newspaper Article Synopsis:**
Scientists at the University of Wisconsin-Madison and at Johns Hopkins University in Balatimore have successfully grown human 'master' cells in the lab. These stem cells are the foundation cells that go on to become bone, nerve, or muscle cells. Both groups used cells from embryos. The potential use for stem cells include growing nerve cells to repair spinal cord injuries, making new bone marrow, or creating islet cells to produce insulin in diabetics.

**Newspaper Article Source:**
Associated Press. Cell-regrowth breakthrough could lead to new therapies. Star Tribune 1998 November 6:A1(col. 1).

**Journal Article Citation:**
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PRIMATE-SCIENCE RESEARCH HIGHLIGHTS
"UW scientist will continue ES cell work with monkeys"
(courtesy Wisconsin Regional Primate Research Center)

The Nov. 6 issue of Science introduces the breakthrough derivation and culture of human embryonic stem cells by James Thomson, Ph.D., at the University of Wisconsin-Madison.

Thomson's accomplishment with human ES cells was not conducted at the Wisconsin Regional Primate Research Center, but rather in a separate lab across campus and with private funding. Nonetheless, the work was in large part the fruit of his extensive experience working with nonhuman primate cells over the past five years.

The research brings us closer to the possibility that researchers may someday be able to genetically engineer ES cells for transplanting in diseased human tissues, where they would be able to grow into healthy cells while remaining free from attack by patients' immune systems.

Although Thomson's breakthrough with human cells received widespread global media attention, it is important to note that his work with monkey ES cells will continue.

"Any human ES cell-based therapies will be completely novel and will require extensive testing to demonstrate safety and efficacy," Thomson says. "The rhesus monkey
and rhesus ES cells will provide an appropriate model for the development of these new transplantation therapies."

Thomson is already establishing collaborations to examine the derivation of neural and blood cells from rhesus ES cells. Transplantation of these specific derivatives could be done in rhesus monkeys to develop treatments for Parkinson's disease or leukemia.

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P-T Research Highlights appears every other week and focuses broadly on research involving non-human primates. Coverage includes biomedicine, behavior, conservation and veterinary science. Please submit highlights for this column to Jordana Lenon, P-T Research Highlights editor, at jlenon@primate.wisc.edu. A 300-word limit and lay-language style are recommended. P-T Research Highlights are supported by a grant from the National Institutes of Health, National Center for Research Resources. 

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# First Derivation Of Human Embryonic Stem Cells Reported In Science

### Geron Holds Worldwide License to a Breakthrough Discovery with Promise for Treating Degenerative Diseases

MENLO PARK, CA -- November 5, 1998 -- Human embryonic stem cells (hES cells) -- unique cells capable of forming all the different cell types and tissues in the body -- have been successfully derived and maintained in culture for the first time by scientists at the University of Wisconsin-Madison, according to an article to be published in the November 6 issue of the journal Science. The research was led by James A. Thomson, VMD, Ph.D., Developmental Biologist at UW-Madison, and was supported by and is licensed to Geron Corporation (NASDAQ:GERN).

"These cells are different from all other human stem cells isolated to date," said Dr. Thomson. "As the source of all cell types, they hold great promise for use in transplantation medicine, drug discovery and development, and the study of human developmental biology."

### The Embryonic Stem Cell

Embryonic stem cells are derived from the inner cell mass of the blastocyst formed during early embryogenesis. Distinguished from all other stem cells, they are pluripotent, able to develop into virtually any and all cells and tissues in the body; and, consistent with their expression of telomerase, self-renewing, a potentially limitless source of cells.

"Geron is focused on developing products to treat age-related degenerative diseases. The availability of hES cells opens extraordinary opportunities for tissue transplantation, and for developing cell and gene therapy products with breakthrough medical potential," said Thomas Okarma, Ph.D., M.D., Geron's vice president of research and development. "Further, Gerons proprietary telomerase technology for prolonging the replicative lifespan of cells derived from hES cells positions the company to potentially supply the preferred cells for transplantation medicine."

### Broad Potential for Medicine, Science and Drug Discovery

Human embryonic stem cells hold enormous promise for transplantation medicine because they can potentially produce unlimited quantities of any cell or tissue in the body. In addition, they may be genetically altered to improve therapeutic value, for instance, to prevent immune system rejection by transplant recipients. Examples of cells that Geron may develop for transplantation therapies include heart muscle cells for use in repairing the tissue damage inflicted by heart attacks, blood forming cells for use in bone marrow transplantation procedures for cancer patients, and nerve cells for use in treating patients with Parkinsons

disease, stroke or Alzheimers disease.

Human embryonic stem cells also represent a new technology for pharmaceutical research and development. Geron plans to generate a variety of different cell types for use in drug screening and toxicology testing. In addition, hES cells can potentially be engineered to create in vivo models of human disease for drug development as a superior alternative to current mouse models. For example, brain neurons derived from hES cells might be engineered to develop the characteristics of Alzheimers disease and used to discover effective drug treatments.

Human embryonic stem cells should also further our understanding of embryonic development with potential applications toward the treatment of infertility and premature pregnancy loss, and the diagnosis and prevention of birth defects.

Finally, hES cells open the door to a new field of research -- the genomics of human developmental biology. Until now, early genetic events in human embryology have been largely inaccessible to direct observation. Research with hES cells may lead to the discovery of novel genes that fundamentally control tissue differentiation. These gene products could result in the development of therapeutic drugs and proteins with potential applications in wound healing, stroke, heart attack and spinal cord injury.

**Collaborations with ES Cell Leaders**

To accomplish its hES cell program objectives, Geron has established collaborations with renowned researchers in the field. The company funds research by and holds worldwide licensing agreements with John D. Gearhart, M.D., Ph.D., professor of gynecology and obstetrics at Johns Hopkins University School of Medicine, and Roger A. Pedersen, Ph.D., professor at the University of California, San Francisco.

Next steps in Geron's hES cell program include the development and optimization of enabling technologies. These include techniques for the production and scale up of hES cells; the identification of cell differentiation factors; techniques for genetically engineering hES cells, and the development of models to test proposed transplant products.

"As we pursue these next steps, Geron recognizes that research in this field should be conducted according to appropriate guidelines," said Ronald W. Eastman, Gerons president and CEO. "This discovery holds great promise for the treatment of a variety of human diseases and conditions. In concert with our Ethics Advisory Board and the Institutional Review Boards of our collaborators, Geron is committed to realizing the vast potential of this technology in a responsible manner."

Geron Corporation is a biopharmaceutical company focusing on discovering and developing therapeutic and diagnostic products based upon the company's understanding of human embryonic stem cells, and of telomeres and telomerase in cells -- fundamental biological platforms underlying cancer and other age-related degenerative diseases.

The company desires to take advantage of the "safe harbor" provision of the Private Securities Litigation Reform Act of 1995. Specifically, the company wishes to alert readers that the matters discussed in this press release constitute forward-looking statements that are subject to certain risks and uncertainties. Actual results may differ materially from the results

anticipated in these forward-looking statements. Additional information on potential factors that could affect the company's results are included in the company's quarterly report on Form 10-Q for the quarter ended June 30, 1998.

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# Culturing Human Embryonic Stem (ES) Cells

In earlier pages, I have described how

- mouse embryonic stem cells can be used to make transgenic mice
- the fusion of a differentiated cell from an adult sheep with an enucleated sheep egg can produce a clone of the cell donor ("Dolly").

The techniques used **in the early steps** of each process have now been achieved with human cells.

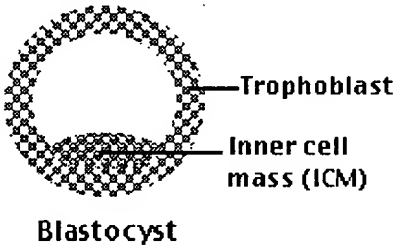
## Human Embryonic Stem (ES) Cells

A research team led by James Thomson of the University of Wisconsin reports in the 6 November 1998 issue of **Science** that they have been able to grow human embryonic stem (**ES**) cells in culture.

At the time of implantation, the mammalian embryo is a **blastocyst**. It consists of the

- **trophoblast** - a hollow sphere of cells that will go on to implant in the uterus and develop into the **extraembryonic membranes**
  - placenta
  - umbilical cord
  - amnion

  Discussion of extraembryonic membranes
- **inner cell mass** (**ICM**) that will develop into the baby.



Blastocyst

The cells of the inner cell mass are considered **pluripotent**; that is, each is capable of producing descendants representing all of the hundreds of differentiated cell types in the newborn baby, including

- ectodermal cells like neurons and skin (epithelial cells)
- mesodermal cells like striated muscle, smooth muscle, cartilage, and bone
- endodermal cells like the intestinal epithelium.

### Their process

- Remove the trophoblast cells from a human blastocyst (these were extras not needed for in vitro fertilization (IVF)
- separate the cells of the **inner cell mass** and culture them on a plate of "feeder" cells (mouse fibroblasts were used)
- isolate single cells and grow them as clones
- Test the clones

### The results

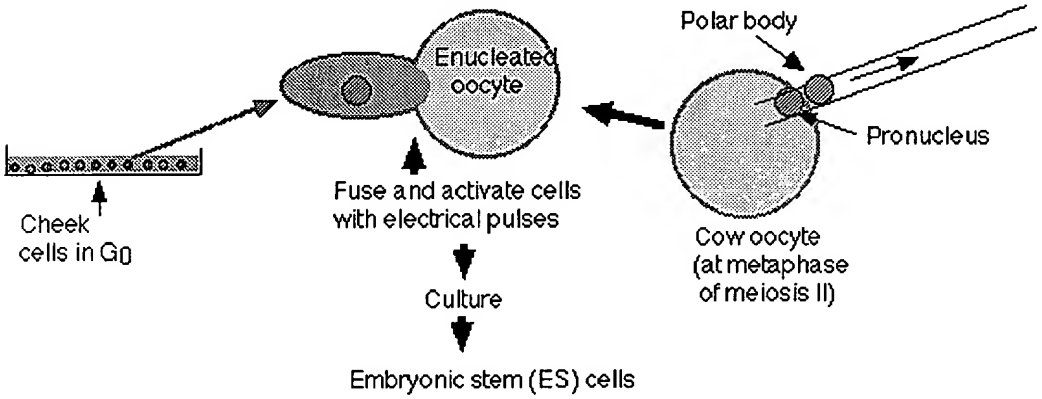
- each successful clone maintained a normal human karyotype (unlike most cultured human cells - HeLa cells, for example)
- these cells had high levels of the enzyme **telomerase**, which maintains normal chromosome length and is characteristic only of cells with unlimited potential to divide ("immortal")

- when injected into **SCID mice**, these cells formed **teratomas**; tumors containing a mix of **differentiated** human cell types, including cells characteristic of
  - **ectoderm**
  - **mesoderm** and
  - **endoderm**

**SCID = severe combined immunodeficiency**.
These mice lack a functioning immune system (have neither T cells nor B cells) and so cannot reject foreign tissue. (Some rare inherited diseases of humans are also called SCID. They produce the same phenotype but the molecular defects are not the same as in the mice.)

# Making ES cells from the differentiated cells of an adult

Researchers at the firm of Advanced Cell Technology (in Worcester, Massachusetts) announced on 11 November, that they had been able to convert **adult** human cells into cultured cells that appear to have the properties of embryonic stem cells; that is, pluripotent and, perhaps, "immortal". (However, they have yet to publish evidence supporting their claim.)




Their process is quite like that used to produce Dolly.

- Cells were removed from an adult human (Jose Cibelli, one of the researchers). Both his cheek cells and his white blood cells were tried.
- Cow oocytes were enucleated
- human cell and enucleated egg were fused using a pulse of electricity
- the resulting cell was grown in culture.

# The Goals of These Achievements

Both these procedures have been used with other animals (mice and sheep). In both those cases, the products were implanted in the uterus of the host animal and grew into a complete animal (cloned mice and Dolly, respectively). Do these workers plan to do the same with their human cells? They assure us that they do not.

So what are their goals?

Human embryonic stem cells have the potential to

- teach us about the process of human embryonic development, its genetic control, etc.
- if the proper signals can be discovered, it may be possible to cause these cells to differentiate along a **particular pathway**, e.g., to form insulin-secreting beta cells of the islets of Langerhans.
    - such cells might be able to replace lost or non-functioning cells in a human patient (e.g., with insulin-dependent diabetes mellitus - IDDM)
    - such cells might be transformed with the DNA needed to express a gene missing in the transplant recipient
    - such cells might be engineered to reduce the risk of transplant rejection.

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